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Differential Role of Insulin Receptor Substrate (IRS)-1 and IRS-2 in L6 Skeletal Muscle Cells Expressing the $Arg^{1152} \rightarrow Gln$ Insulin Receptor*

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In L6 muscle cells expressing the $Arg^{1152} \rightarrow Gln$ insulin receptor (Mut), basal tyrosine phosphorylation of insulin receptor substrate (IRS)-1 was increased by 35% compared with wild-type cells (WT). Upon exposure to insulin, IRS-1 phosphorylation increased by 12-fold in both the Mut and WT cells. IRS-2 was constitutively phosphorylated in Mut cells and not further phosphorylated by insulin. The maximal phosphorylation of IRS-2 in basal Mut cells was paralleled by a 4-fold increased binding of the kinase regulatory loop binding domain of IRS-2 to the $Arg^{1152} \rightarrow Gln$ receptor. Grb2 and phosphatidylinositol 3-kinase association to IRS-1 and IRS-2 reflected the phosphorylation levels of the two IRSs. Mitogen-activated protein kinase activation and [^{3}H]thymidine incorporation closely correlated with IRS-1 phosphorylation in Mut and WT cells, while glycogen synthesis and synthase activity correlated with IRS-2 phosphorylation. The $Arg^{1152} \rightarrow Gln$ mutant did not signal Shc phosphorylation or Shc-Grb2 association in intact L6 cells, while binding Shc in a yeast two-hybrid system and phosphorylating Shc *in vitro*. Thus, IRS-2 appears to mediate insulin regulation of glucose storage in Mut cells, while insulin-stimulated mitogenesis correlates with the activation of the IRS-1/mitogen-activated protein kinase pathway in these cells. IRS-1 and Shc-mediated mitogenesis may be redundant in muscle cells.

Insulin induces proliferative and metabolic responses in several different tissues (1). The biological effects of insulin in its target cells are initiated by binding and activating tyrosine kinase receptors (2), followed by phosphorylation of intracellular protein substrates (2, 3). The phosphorylated substrates, in turn, bind SH2[1] domain-containing proteins (2, 3) further propagating receptor signal into at least two major transduction routes. These pathways include the Ras/mitogen-activated protein kinase (MAP kinase) cascade and the PI 3-kinase system (2, 3), and convey insulin signal to the final cytoplasmic and nuclear effectors.

Insulin receptor substrate-1 (IRS-1) is an important intracellular substrate for the insulin receptor kinase (3). IRS-1 features at least 7 tyrosine residues undergoing rapid phosphorylation upon insulin receptor activation (4) and providing binding sites for at least six distinct SH2 proteins (2–5). Tyrosine-phosphorylated IRS-1 binds the SH2 domain in the p85 regulatory subunit of PI 3-kinase (6, 7) inducing several metabolic responses (8, 9). Phosphorylated IRS-1 also interact with the Grb2·SOS complex, activating $p21^{ras}$, the MAP kinase cascade and mitogenesis (10–12). Similar to IRS-1, the oncoprotein Shc is tyrosine phosphorylated by the insulin receptor followed by binding to Grb2·SOS and MAP kinase activation (13, 14). Therefore, IRS-1 and Shc represent distinct links conveying insulin signal through the MAP kinase machinery and evoking proliferative responses. In Rat1 fibroblasts expressing human insulin receptors (15), as well as in other cells, most SOS guanylnucleotide exchange activity co-precipitated with Shc rather than IRS-1, suggesting a major role of Shc in Ras activation by insulin. However, in 32-D cells expressing insulin receptors, MAP kinase activation by insulin requires Grb2 binding to IRS-1 (16). Thus, the relative role of IRS-1 and Shc in mediating proliferative response through the Ras·MAP kinase cascade remains controversial and may be cell- and tissue-specific.

IRS-2 is another cellular substrate for the insulin receptor kinase, which has been more recently identified in liver and skeletal muscle cells (17). IRS-2 shares many structural features with IRS-1 (2, 3, 18). Like IRS-1, insulin receptor-phosphorylated IRS-2 binds to both PI 3-kinase and Grb2 (2, 3, 19). In IRS-1 knock-out mice, IRS-2 phosphorylation is substantially increased as compared with the wild-type animals, suggesting that this increase may compensate for the lack of IRS-1 thus improving insulin action on glucose metabolism (20). Evidence has also been reported that IRS-2 mediates insulin-stimulated translocation of GLUT4 in a fashion similar to IRS-1(2, 3). However, whether each of these two substrates specializes in mediating certain insulin bioeffects or whether they are largely redundant into the cells has not been conclu-

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[1] The abbreviations used are: SH2, Src homology 2; IR, insulin receptor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; Ab, antibody; WT, wild-type; MAP, mitogen-activated protein; PI, phosphatidylinositol; IGF-I, insulin-like growth factor-I; KRLB, kinase regulatory loop binding; PTB, phosphotyrosine binding; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

TABLE I
Basal and insulin-stimulated IRS phosphorylation and glycogen synthase activity in L6 expressing endogenous and wild-type human insulin receptor

IRS phosphorylation and glycogen synthase activity were determined as described in Materials and Methods. Each value represents the mean ± S.D. of at least 3 independent experiments.

Cell line	IRS-1 phosphorylation			IRS-2 phosphorylation			GS activity		
	Basal	Maximal	ED_{50}	Basal	Maximal	ED_{50}	Basal	Maximal	ED_{50}
	arbitrary units		*nM*	*arbitrary units*		*nM*	*% of I form*		*nM*
L6	1.5 ± 0.1	10 ± 0.9	3.2	3.8 ± 0.4	19 ± 1.8	2.4	14 ± 1.8	25 ± 1.6	1.0
WT_1	1.6 ± 0.2	14 ± 1.2	1.0	5.0 ± 0.3	20 ± 1.8	0.8	14.5 ± 1.3	27 ± 1.9	0.1
WT_3	1.5 ± 0.1	12.5 ± 1.0	1.5	4.0 ± 0.4	19 ± 2.0	1.2	14 ± 1.5	24 ± 1.7	0.6

sively established. Additionally, it remains unclear whether variability exists in the relative role of IRS-1 and IRS-2 in mediating insulin action in the different target tissues.

In the present report, we have studied insulin signaling in L6 skeletal muscle cells expressing the IR^{1152} insulin receptor. This mutant receptor maximally activates metabolic responses, preventing further insulin stimulation, but normally transduces insulin mitogenic signals. In addition, as we show in this work, IR^{1152} differentially phosphorylates IRS-1, IRS-2, and Shc, enabling us to address their relative function in mediating proliferative and metabolic signals in skeletal muscle, a major insulin-responsive tissue.

MATERIALS AND METHODS

General Procedures—L6 cell clones expressing 3×10^3 insulin receptors were selected and transfected with either the mutant IR^{1152} or the wild-type insulin receptors and have been previously characterized and described (21). In the present study, two clones of transfected cells expressing 3.2×10^4 or 9×10^3 wild-type IRs/cell and 3.1×10^4 or 9.5×10^3 mutant IRs/cell were used. At these low levels, overexpression of wild-type receptors in L6 as in other cells (22) is accompanied by little change in maximal insulin effects on most signaling events and cell responses. However, a 3–10-fold decrease in the ED_{50} for insulin effect on IRS-1 and IRS-2 phosphorylation and on glycogen synthase activity and thymidine incorporation could be consistently detected (Table I). These cells express fully functional GLUT4 transporters (21, 23–27). The antibodies against phosphotyrosine, IRS-1, IRS-2, Shc, Grb2, MAP kinase, and p85 PI 3-kinase were purchased from either Upstate Biotechnology Inc. (Lake Placid, NY) or Santa Cruz Biotechnology (Santa Cruz, CA). Media and sera for tissue culture were from Life Technologies, Inc., electrophoresis and Western blot reagents from Bio-Rad, and protein A beads (Trisacryl) from Pierce. All other chemicals were from Sigma. The yeast strain L40 (*MAT a, trp1, leu2, his3, LYS2::lexA-His3, URA3::lexA-lacZ*) and the yeast expression plasmids pBTM116 were obtained from A. Vojtek (Fred Hutchinson Cancer Research Center, Seattle, WA). The plasmid pACTII was provided by Dr. S. Elledge (Baylor College of Medicine, Houston, TX) and the human insulin receptor cDNA by Dr. S. Gammeltoft (Bispebjerg Hospital, Copenhagen, Denmark). The two-hybrid plasmids, the L40 strains, and the cDNA constructs used in this study have been described previously (28, 29).

Detection of IRS-1, IRS-2, Shc, Grb2, and p85—Cells were stimulated with 100 nM insulin for 5 min at 37 °C as indicated, and lysed in 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (TAT buffer). Lysates were then incubated with IRS-1, IRS-2, or Shc Abs for 18 h at 4 °C, and immunocomplexes precipitated with protein A beads, solubilized in Laemmli buffer, separated by PAGE, and Western-blotted as described in Ref. 30. Blots were probed with phosphotyrosine, Grb2, or p85 Abs as in Ref. 30 and revealed with ^{125}I-labeled protein A and autoradiography. Quantitation was achieved by laser densitometry of the bands.

Determination of PI 3-Kinase and MAP Kinase Activities—For PI 3-kinase, cells were stimulated with 100 nM insulin for 15 min at 37 °C as indicated, and solubilized for 40 min at 4 °C in 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (TAN buffer). Aliquots of the lysates were precipitated with IRS-1 or IRS-2 Abs coupled to protein A Sepharose for 2 h at 4 °C. PI 3-kinase activity was determined in pellets as described in Ref. 31.

For MAP kinase assays, cells were lysed in 50 mM β-glycerol phosphate, 10 mM Hepes, pH 8.0, 70 mM NaCl, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride. Determination of MAP kinase activity in the lysates was performed as described in Ref. 32. Briefly, 10 μl of the lysates (10 μg of cell protein) were incubated with 5 μg of myelin basic protein for 15 min at 25 °C in a final volume of 25 μl of 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 10 mM $MgCl_2$, 40 mM [γ-^{32}P]ATP. The reaction was terminated by addition of 4× Laemli buffer and myelin basic protein phosphorylation determined by PAGE separation, followed by quantitation of radioactivity in the excised bands.

Glycogen Accumulation, Glycogen Synthase, and Thymidine Incorporation Assays—Cells were exposed to 50 nM wortmannin for 30 min at 37 °C before stimulation with insulin (20 min at 37 °C), as indicated. Glycogen content of the cells was determined as described previously (21). Glycogen synthase activity was assayed by a modification of the method by Thomas *et al.* (33). Briefly, cells were preincubated in Hepes buffer, pH 7.8, for 3 h at 37 °C before the assay. The cells were further incubated with 50 nM wortmannin and then stimulated with 100 nM insulin (in the presence or the absence of wortmannin) as indicated, resuspended in ice-cold 100 nM NaF, 10 mM EDTA, and sonicated for 10 s at 300 watts. Cells were then centrifuged for 10 min at 2,000 rpm, and 20-μl aliquots of the supernatants (20 μg of cell proteins) incubated with 60 μl of a reaction mixture containing 40 mM Tris-HCl, pH 7.8, 25 mM NaF, 20 mM EDTA, 10 mg/ml glycogen, 7.2 nM uridine 5′-diphosphate-glucose, in the absence or the presence of 6.7 mM glucose 6-phosphate. The incubation was prolonged for 20 min at 30 °C and terminated by spotting on filter paper followed by precipitation with ice-cold ethanol. Precipitated radioactivity was quantitated in a Beckman scintillation counter. Enzyme activity was expressed as a percentage of the glucose 6-phosphate-independent form (21).

For thymidine incorporation, six-well plates were seeded with 10^5 cells/well. After 24 h, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 0.05% bovine serum albumin and no serum, incubated for 24 h, and than further incubated for 16 h with the same medium supplemented with 50 mM wortmannin and 100 nM insulin as indicated. [^{3}H]Thymidine was then added at a specific activity of 500 nCi/ml, and incorporation into DNA was quantitated as described previously (34).

Transformation of Yeast Strains and β-Galactosidase Assay—Plasmid DNA transformations were performed using the lithium acetate method of Gietz *et al.* (35). Cotransformants were selected on Trp^-, Leu^- plates. The transformants were tested for β-galactosidase activity by liquid culture assays using the substrate *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (36).

Insulin Receptor Interaction with IRS-2 Fusion Proteins and Phosphorylation of Immobilized Substrates—Construction of the IRS-2 fusion proteins, partial purification of insulin receptors, and precipitation of autophosphorylated insulin receptors by IRS-2 fusion proteins were performed as described in Ref. 37. To analyze the phosphorylation of insulin receptor substrates *in vitro*, parental L6 myotubes were lysed with TAT buffer and precipitated with IRS-1, IRS-2, or Shc antibodies. Precipitated proteins were then incubated with protein A-Sepharose and the immobilized proteins further incubated with wild-type or mutant insulin receptors (250 fmol/assay). Phosphorylation reactions were initiated by adding 2 mM CTP, 2 μM ATP, 10 mM HEPES, pH 7.4, 0.02% Triton X-100, 5 mM $MnCl_2$, 7 mM $MgCl_2$, 0.02 μM [γ-^{32}P]ATP (final concentrations), and prolonged for 30 min at 22 °C. Phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography.

RESULTS

IRS Phosphorylation in L6^{1152} Myotubes—Lysates were prepared from L6 skeletal muscle cells expressing the constitu-

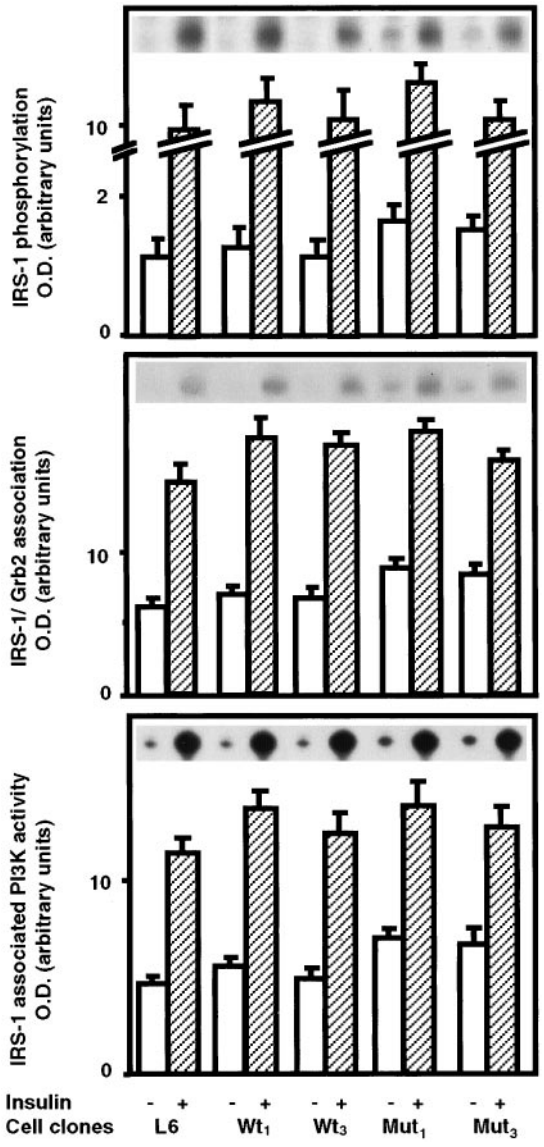

FIG. 1. **IRS-1 phosphorylation and association to SH2 proteins in Mut cells.** Different clones of L6 myotubes, either parental cells (L6; 3.1×10^3 IR/cell) or cells expressing human wild-type (WT_1 and WT_3; 3.2×10^4 and 9×10^3 IR/cell, respectively) or mutant insulin receptors (Mut_1 and Mut_3; 3.1×10^4 and 9.5×10^3 IR/cell, respectively) were incubated in the absence or the presence of 100 nM insulin as described under "Materials and Methods." Cell lysates were precipitated with IRS-1 Abs and then immunoblotted with phosphotyrosine (*top*) or Grb2 (*middle*) Abs. Blotted proteins were revealed with ^{125}I-protein A and autoradiography. PI 3-kinase activity in IRS-1 precipitates (*bottom*) was assayed as described under "Materials and Methods." Quantitation of both the IRS-1·Grb2 bands and PIP spots was achieved by laser densitometry. In each panel, the *bars* represent the mean values ± S.D. from at least three independent experiments. A representative experiment is shown in each *inset*. Based on *t* test analysis, the differences in basal IRS-1 phosphorylation in Mut_1 and Mut_3 *versus* control cells were significant at the $p < 0.001$ and $p < 0.05$ levels, respectively. The increased basal IRS-1 association with Grb2 and PI 3-K activity in Mut_1 and Mut_3 cells was significant at the $p < 0.05$ (Grb2 association) and $p < 0.001$ (PI 3-K activity) levels.

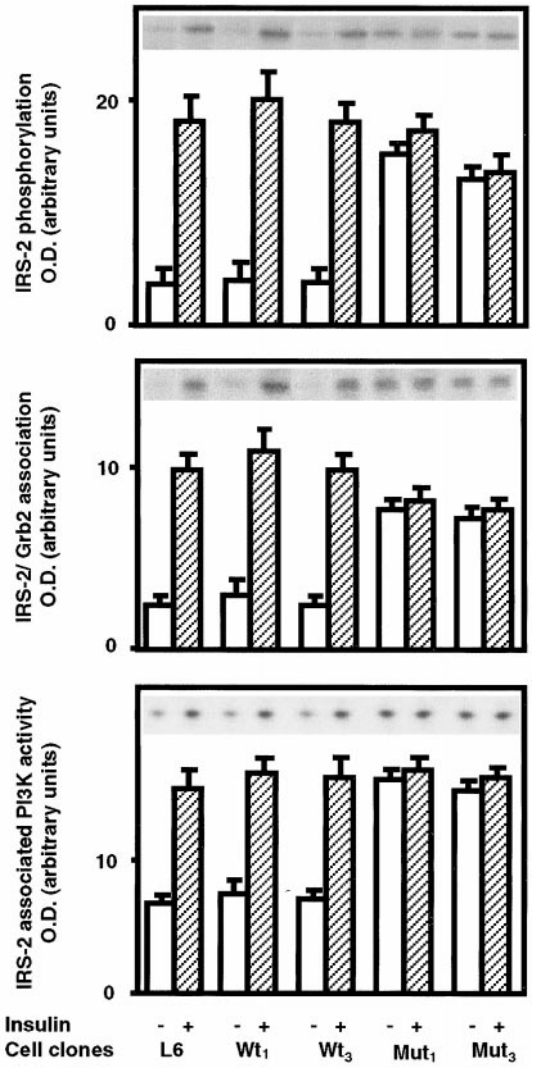

FIG. 2. **IRS-2 phosphorylation and association to SH2 proteins in Mut cells.** WT and Mut cells were incubated with insulin as described in the legend to Fig. 1, immunoprecipitated with IRS-2 Abs, and then immunoblotted with phosphotyrosine (*top panel*) or Grb2 (*middle panel*) Abs or assayed for PI 3-kinase activity (*bottom panel*). Detection and quantitation was achieved as in the experiment shown in Fig. 1. In each panel, the *bars* represent the mean values ± S.D. from at least three independent experiments. The difference in basal values between Mut and control cells are statistically significant ($p < 0.001$). A representative experiment is shown in each *inset*.

tively active $Arg^{1152} \rightarrow$ Gln insulin receptors (Mut cells) (21). Immunoprecipitation of these lysates (Mut_1 cell clone, 3.1×10^4 IR/cells) with IRS-1 Abs followed by blotting with phosphotyrosine antibodies (Tyr(P) Abs) revealed a slight increase (35%, $p < 0.001$) in basal IRS-1 tyrosine phosphorylation as compared with lysates from control cells, either those expressing a comparable number of wild-type hIRs or those from parental cells (WT, L6 cells, respectively; Fig. 1, *top panel*). A 20% basal increase in IRS-1 phosphorylation was also detectable in cells expressing smaller number of mutant receptors (Mut_3; 9×10^3 receptors/cell; $p < 0.05$). Exposure to insulin produced a similar 10–12-fold increase in IRS-1 phosphorylation in all of the cell lines.

Tyrosine-phosphorylated IRSs bind different SH2 proteins, including the Grb2 adaptor and the p85 regulatory subunit of PI-3 kinase, which propagate insulin signal (2, 3, 5, 19). In parallel with IRS-1 phosphorylation, Grb2 co-precipitation with IRS-1 was also slightly increased in Mut as compared with WT cells (Fig. 1, *middle panel*, $p < 0.05$). Likewise, recovery of PI 3-kinase activity in the IRS-1 immunoprecipitates was 20–30% increased in cells expressing IR^{1152} (Fig. 1, *bottom panel*, $p < 0.001$). After insulin addition to both WT and Mut cells, Grb2 association with IRS-1 and IRS-1-bound PI 3-kinase were stimulated by almost 3-fold.

At variance with IRS-1, basal phosphorylation of IRS-2 in Mut cells was constitutively increased by almost 4-fold as compared with control cells (Fig. 2, *top panel*). These phosphoryl-

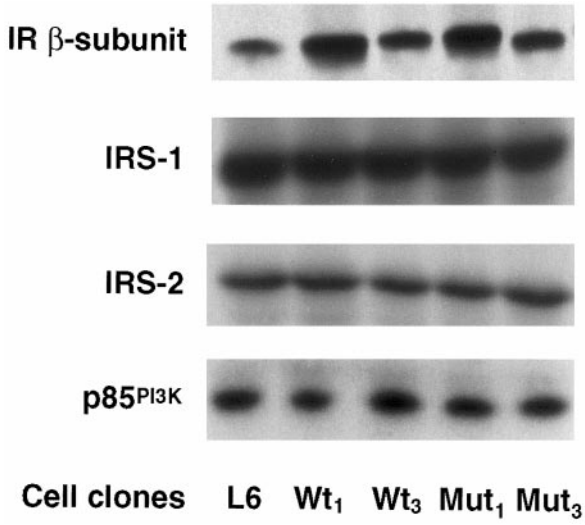

FIG. 3. **IR, IRS, and PI 3-kinase levels in Mut cells.** The myotubes were lysed and cell proteins separated by SDS-PAGE as described under "Materials and Methods." Proteins were then blotted using specific IR, IRS-1, IRS-2, or p85 antibodies and revealed by ^{125}I-protein A and autoradiography. The autoradiographs shown are representative of at least three independent experiment for each of the proteins analyzed.

ation levels were comparable to those of wild-type cells after maximal insulin stimulation. Exposure of Mut cells to insulin concentrations up to 100 nM did not further increase IRS-2 tyrosine phosphorylation, however, while increasing that in control cells by 5-fold. Grb2 co-precipitation with IRS-2 and recovery of PI 3-kinase activity in IRS-2 immunoprecipitates also increased by 4- and 2-fold, respectively, in the insulin-stimulated control cells, while exhibiting high basal levels and no insulin sensitivity in the mutant cells (Fig. 2, *middle* and *bottom panels*). It appeared therefore that the IR^{1152} mutant phosphorylated IRS-1 and 2 and induced their binding to SH2 intracellular proteins differentially both in the absence and in the presence of insulin. This effect could not be ascribed to differences in IRS-1, IRS-2, PI 3-K (Fig. 3), or Grb2 (Fig. 7) levels since these were comparable in all of the cell clones analyzed. The relative levels of endogenous, wild-type, and mutant insulin receptors in the cell clones are also shown in Fig. 3. Data similar to those in the L6 cells were also obtained with NIH-3T3 fibroblasts expressing the mutant and wild-type insulin receptors (data not shown). As in the intact cells, *in vitro* phosphorylation of immobilized IRS-2 by affinity-purified IR^{WT} showed a 3-fold increase upon insulin addition, while phosphorylation of immobilized IRS-2 by basal IR^{1152} exhibited a 2.8-fold increase as compared with the wild-type receptors and was not further stimulated by insulin (Fig. 4, *top panel*). *In vitro* phosphorylation of immobilized IRS-1 by the mutant receptors showed a slight 30% basal increase compared with that by the wild-type receptor ($p < 0.05$) and was fully phosphorylated upon insulin stimulation, suggesting that the IR^{1152} mutation mainly enhances the interaction of the receptor with IRS-2. To examine this possibility, we analyzed the ability of IR^{1152} to bind the kinase regulatory loop binding (KRLB) domain of IRS-2. This insulin receptor binding region has been previously reported to be unique to IRS-2 and not present in IRS-1 (38). IR^{WT} and IR^{1152} were incubated with immobilized GST-IRS-2-KRLB and KRLB-bound receptors were then immunoblotted with insulin receptor antibodies. As shown in Fig. 4 (*middle panel*), almost no wild-type receptors bound to the KRLB domain in basal conditions while insulin activation of these receptors determined a 4-fold increase in KRLB binding. At variance, with the IR^{1152} mutant, binding to the KRLB domain was already maximal in the absence of insulin and did

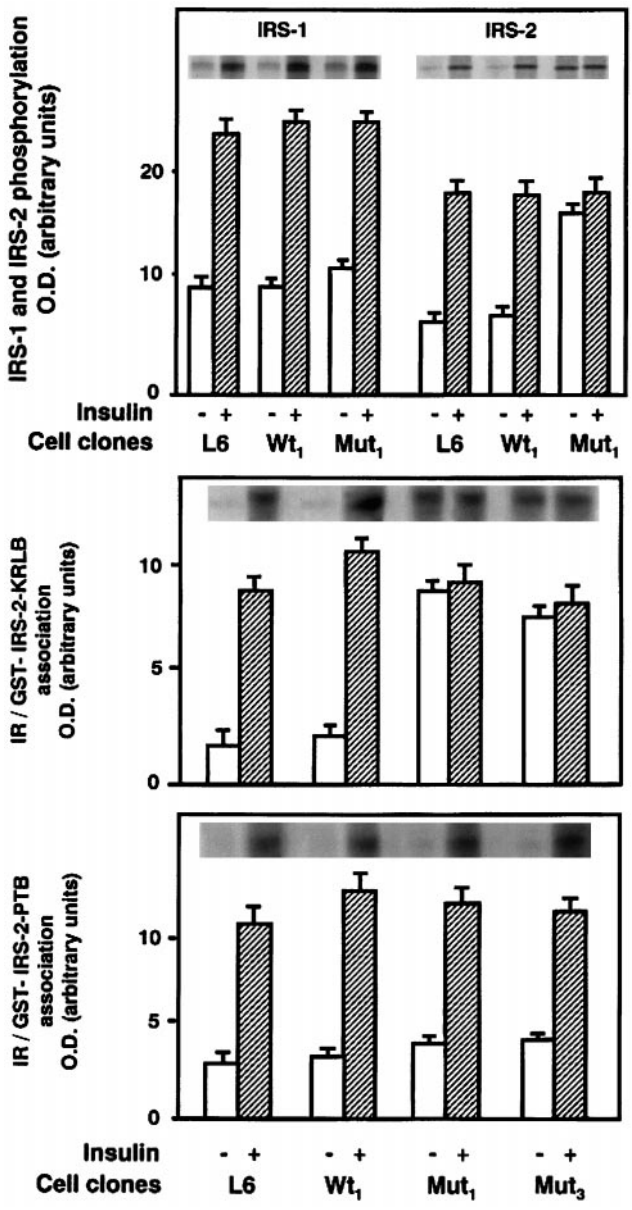

FIG. 4. ***In vitro* interaction of IR^{1152} with IRSs.** *Top*, substrates phosphorylation by IR^{1152} IRS-1, and IRS-2 from L6 parental myotubes were immobilized on Sepharose beads and phosphorylated *in vitro* as described under "Materials and Methods," using insulin receptors purified from parental (L6), WT, or Mut cells as indicated. Phosphorylated proteins were then separated and analyzed by SDS-PAGE. *Middle* and *bottom panels*, precipitation of IR^{1152} by IRS-2 fusion proteins. Purified wild-type and mutant insulin receptors were incubated with immobilized KRLB or PTB domains of IRS-2 as described under "Materials and Methods," immunoblotted with specific receptor antibodies, and revealed by ECL and autoradiography. Each *bar* represents the mean ± S.D. of values from three independent experiments. A representative autoradiograph is shown in each *inset*.

not further increase upon insulin addition. In this same assay, there was no difference in IR^{1152} and IR^{WT} binding to the IRS-2 PTB domain (Fig. 4, *bottom panel*). Thus, the constitutive IR^{1152} kinase activity toward IRS-2 is accompanied by an enhanced binding of the mutant receptor to the KRLB domain of IRS-2, independent of insulin.

Induction of Metabolic and Proliferative Responses through the IR^{1152} Receptor—While tyrosine phosphorylation is known to represent a prerequisite for enabling IRS-1 and IRS-2 to transduce insulin signal downstream the receptor, their relative role in inducing metabolic responses in target tissues re-

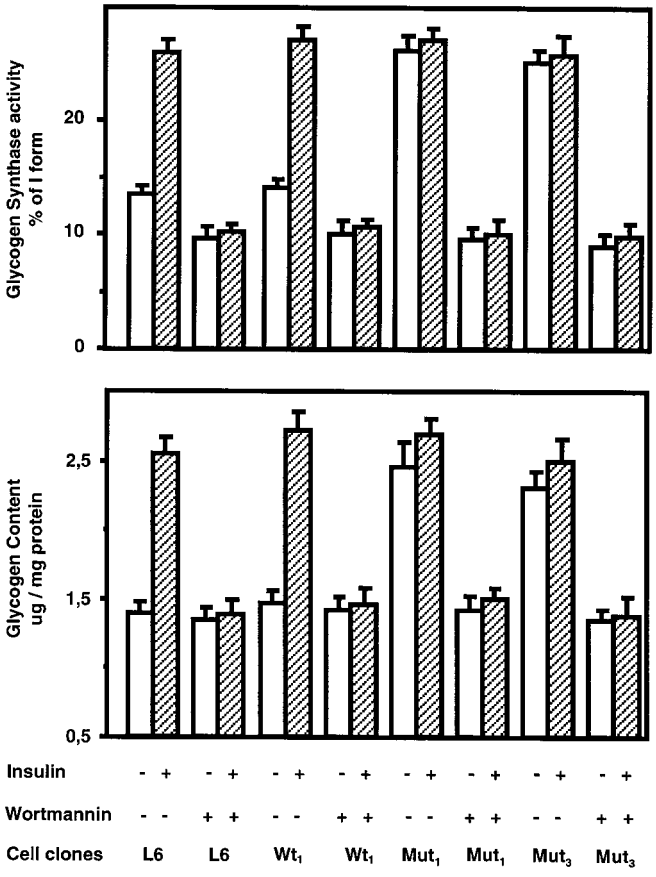

FIG. 5. **Glycogen synthase activity and glycogen content in Mut cells.** The cells were preincubated with 50 nM wortmannin for 30 min and then further exposed to 100 nM insulin for 30 min (glycogen synthase) or 12 h (glycogen content). Glycogen synthase activity (*top panel*) and glycogen content (*bottom panel*) in cell extracts were determined as described under "Materials and Methods." Each value is the mean ± S.D. of duplicate determinations in four (glycogen synthase) and three (glycogen content) experiments. The differences in basal values between Mut and control cells are statistically significant ($p < 0.001$).

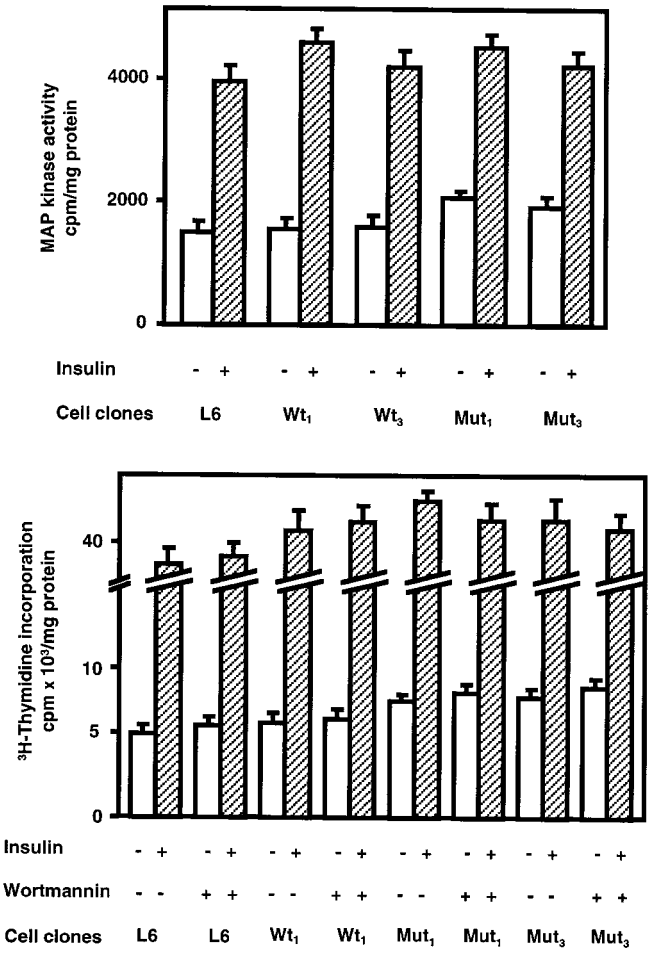

FIG. 6. **MAP kinase and thymidine incorporation in Mut cells.** Different clones of L6 myoblasts expressing either human wild-type (WT_1 and WT_3) or mutant insulin receptors (Mut_1 and Mut_3) were preincubated with 50 nM wortmannin and then exposed to 100 nM insulin. MAP kinase activity in cell lysates and [^{3}H]thymidine incorporation into DNA were determined as described under "Materials and Methods." Each value is the mean ± S.D. of duplicate determinations in six (MAP kinase) and five (thymidine incorporation) experiments.

mains unsettled (20). As we previously reported in the L6 myotubes (21), expression of IR^{1152} constitutively activated glycogen synthase blocking insulin stimulation of the enzyme. Glycogen content in these cells was also maximal in the basal state and not further stimulated by insulin (Fig. 5). In Mut cells, both glycogen synthase and glycogen accumulation were returned to levels similar to those detected in basal control cells after incubation with the PI 3-kinase inhibitor wortmannin. Wortmannin also blocked insulin-stimulated activation of glycogen synthase and glycogen accumulation in WT cells, indicating that PI 3-kinase mediated both the induction of these responses through the wild-type IR and through the constitutively active mutant (Fig. 5).

Activation of the MAP kinase system by the Grb2·SOS complex represents a major mechanism conveying IR mitogenic signals to the nucleus (11). In the Mut cells, MAP kinase activity exhibited a modest basal increase (30%, $p < 0.001$) as compared with the control cells (Fig. 6, *top panel*). [^{3}H]Thymidine incorporation into DNA also exhibited a slight basal increase in the mutant-expressing cells ($p < 0.05$; Fig. 6, *bottom panel*). Insulin addition rapidly stimulated MAP kinase activity in the Mut and control cells by 2.5- and 3-fold, respectively, and increased [^{3}H]thymidine incorporation into DNA by more than 6-fold in both cell lines. At variance from glycogen synthesis and glycogen synthase activity, insulin-stimulated thymidine incorporation was not affected by preincubation of the cells with wortmannin. Thus, in the Mut cells, glucose storage reflected IRS-2 but not IRS-1 phosphorylation and was blocked by PI 3-kinase inhibition. In contrast, proliferative responses better correlated with IRS-1 than with IRS-2 phosphorylation levels and are independent of PI 3-kinase activity.

Shc Phosphorylation in IR^{1152}-expressing Cells—In most cells, phosphorylation of the IR substrate Shc and its association to the Grb2·SOS complex is considered to represent a major and wortmannin-independent pathway transducing insulin mitogenic signals (15). Whether the Shc route is necessary or redundant for insulin-induced mitogenesis is currently unknown. Based on immunoprecipitation of Shc followed by blotting with Tyr(P) Ab, phosphorylation of $p52^{shc}$ was detectable in both the control and the Mut cells (Fig. 7, *middle panel*). However, Shc was not constitutively phosphorylated in Mut cells. In addition, insulin increased Shc phosphorylation by 3-fold in the wild-type cells, but had almost no effect in several mutant clones. The levels of Shc and Grb2 were identical in control and Mut cells (Fig. 7, *top panel*). Insulin had also no effect on Grb2 association with Shc in Mut cells, although increasing that in the WT by 2-fold (Fig. 7, *bottom panel*). Therefore, in the IR^{1152}-expressing cells, insulin-induced mitogenesis occurred in the absence of Shc phosphorylation and its subsequent Grb2 association.

Shc interaction with the insulin receptor has been reported

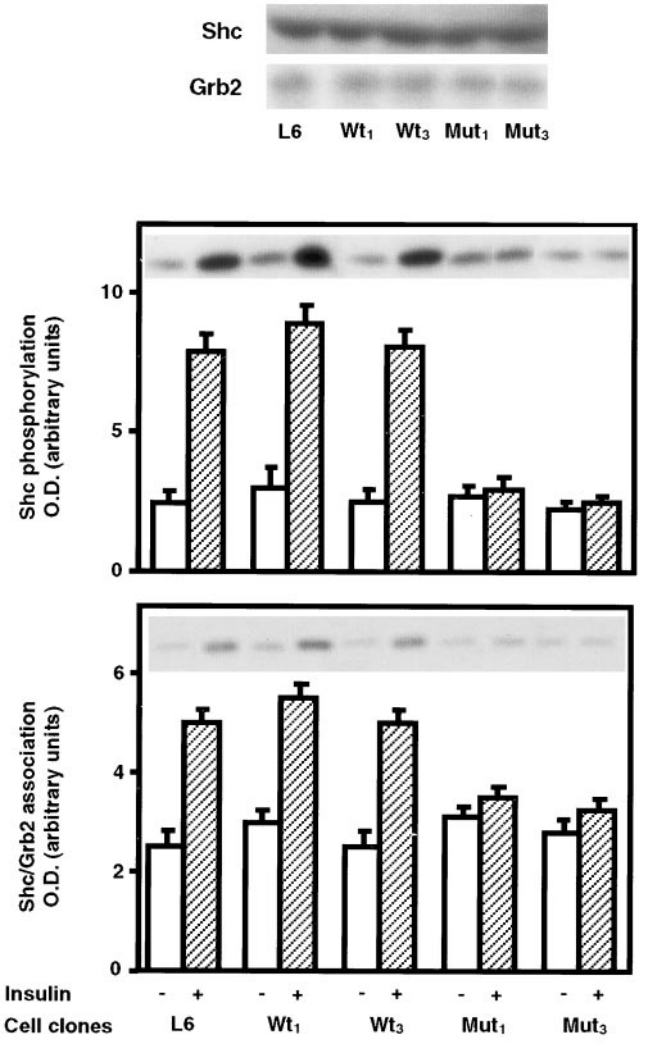

FIG. 7. **Shc phosphorylation and Grb2 association in Mut cells.** The L6 myoblasts were incubated in the absence or the presence of 100 nM insulin as indicated. Cell lysates were precipitated with Shc Abs and then immunoblotted with phosphotyrosine (*middle panel*) or Grb2 (*bottom panel*) Abs. Blotted proteins were revealed with [^{125}I]protein A and autoradiography. Quantitation of the bands was achieved by laser densitometry of the autoradiographs. In each panel, the *bars* represent the mean values ± S.D. from at least three independent experiments. A representative experiment is shown in each *inset*. For detection of total cellular levels of Shc and Grb2 (*top panel*), equal amounts of solubilized cell proteins were separated by SDS-PAGE and blotted with Shc or Grb2 Abs. A representative experiment is shown.

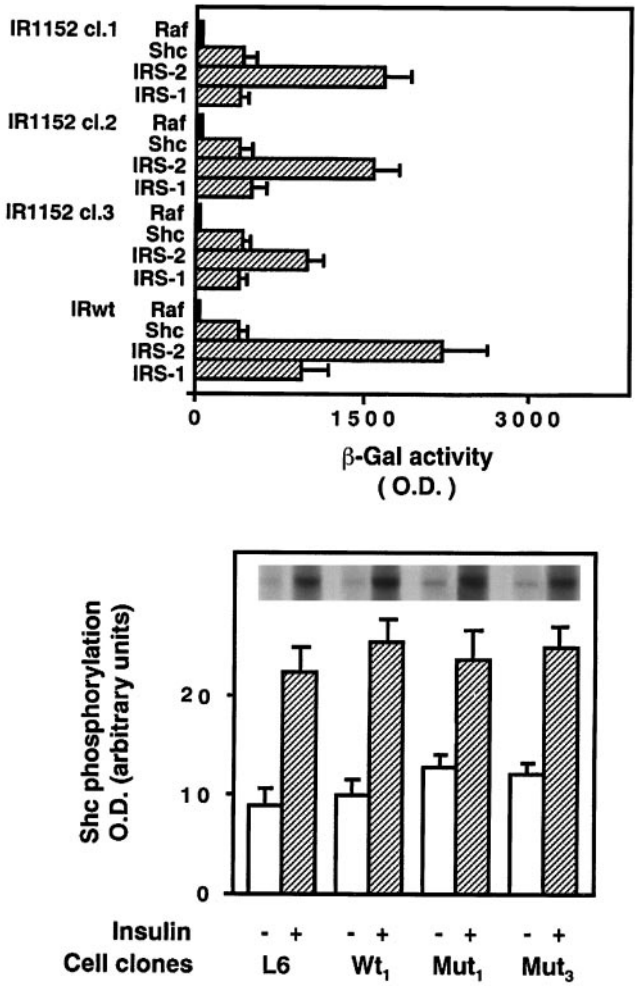

FIG. 8. **Analysis of IR^{1152} interaction with Shc and other insulin receptor substrates.** *Top panel*, measurement of the β-galactosidase activity in transformed yeast is shown. The yeast reporter strain L40 was co-transformed with a plasmid encoding the LexA DNA binding domain-IRβ in combination with a plasmid encoding GAD-Shc, GAD-IRS-1, or GAD-IRS-2. Transformants were isolated on selective plates. Activation of the LexA-LacZ reporter gene was monitored by measuring β-galactosidase activity in cell lysates using *o*-nitrophenyl-β-D-galactopyranoside as substrate. The activities are expressed in Miller's units (36) and are the average ± S.D. of values obtained with samples prepared from three independent transformants. Bottom, Shc phosphorylation by IR^{1152}. Shc from L6 parental myotubes were immobilized on Sepharose beads and phosphorylated *in vitro* as described under "Materials and Methods," using insulin receptors purified from either the parental cells or the WT and Mut cells as indicated. Phosphorylated proteins were then separated by SDS-PAGE and revealed by autoradiography. Each *bar* represents the mean ± S.D. of at least three independent experiments. A representative autoradiograph is shown in the *inset*.

to depend on a PTB domain homologous to that of IRS-1 and IRS-2 (39). Since the IR^{1152} mutant normally binds to the PTB domain of IRS-2, we sought to investigate further the ability of this mutant receptor to bind Shc in a yeast two-hybrid analysis. Shc full-length cDNA was fused to the Gal4 activation domain, whereas the catalytically active cytoplasmic portion of the insulin receptor (including the juxtamembrane region) was fused to the LexA DNA binding domain. Gal4-fused IRS-1 and IRS-2 full-lengths and Raf full-length were included as positive and negative controls, respectively. As shown in Fig. 8 (*top panel*), Shc interacted with IR^{1152} as well as with the wild-type insulin receptor in the yeast two-hybrid assay. Consistent with the data shown in the previous sections of this report, IRS-1 and IRS-2 also interacted with the mutant and the wild-type insulin receptors, while Raf did not. *In vitro*, IR^{1152} elicited a 30% increase in the basal phosphorylation of Shc as compared with IR^{WT} ($p < 0.05$; Fig. 8, *bottom panel*). Insulin addition induced a further 70% increase in Shc phosphorylation by the mutant receptor, similar to that measured with receptors from control cells (difference not statistically significant). Thus, the data indicated that the lack of Shc phosphorylation in intact Mut cells did not directly result from an effect of the IR^{1152} mutation on IR^{1152}-Shc binding or phosphorylation. Alternatively, we postulated that the lack of *in vivo* phosphorylation might be caused by the abnormal intracellular routing, which characterizes the IR^{1152} receptors (40). To test this hypothesis, we analyzed Shc phosphorylation upon 24-h preincubation of the cells with TPA. This treatment shifts the internalized insulin receptors toward the retroendocytotic rather than the degradative compartment, thus mimicking IR^{1152} routing in the L6 myotubes as well as in the NIH-3T3 fibroblasts (41). As shown in Fig. 9 (*top panel*), TPA preincubation of control cells, reduced

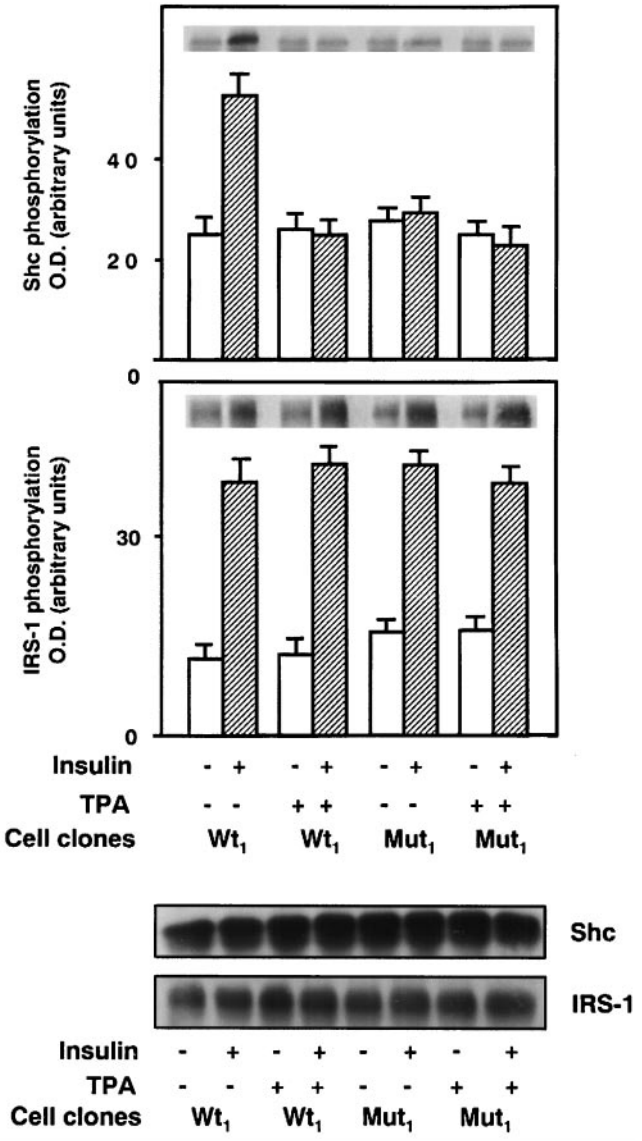

FIG. 9. **Effect of TPA on insulin-stimulated Shc phosphorylation in L6 cells.** *Top*, L6 parental myotubes and cells expressing WT and IR^{1152} mutant receptors were incubated with 1 μM TPA for 24 h and further stimulated with 100 nM insulin for 10 min at 37 °C as indicated. Shc phosphorylation in the intact cells was then analyzed as described in the legend to Fig. 7. Each *bar* represents the mean value ± S.D. of at least three independent determinations. A representative autoradiograph is shown in the *inset*. *Bottom*, to verify the total levels of Shc and IRS-1 upon TPA treatment, aliquots of the cell lysates were subjected to SDS-PAGE and blotted with Shc or IRS-1 specific antibodies as indicated.

the insulin-stimulated phosphorylation of Shc to levels comparable to those measured in untreated Mut cells with no change in the total Shc levels of the cells (Fig. 9, *bottom panel*). TPA did not further reduce the insulin-stimulated Shc phosphorylation in the Mut cells. At variance with Shc phosphorylation, preincubation with TPA elicited no change in IRS-1 phosphorylation by either the wild-type or the IR^{1152} receptors (Fig. 9, *middle panel*).

DISCUSSION

Insulin evokes a wide range of metabolic and mitogenic responses by binding tyrosine kinase receptors and phosphorylating tyrosines on several intracellular protein substrates (1–3). These include IRS-1, IRS-2, and Shc. While the relevance of these IR substrates in propagating insulin signal has been well established (2, 3), the specific role of each of them as well as the extent to which they are redundant or complementary is less clear (3). In addition, IRS-1, IRS-2, and Shc may feature tissue specificity in the major targets for insulin action, muscle, liver, and adipose tissues (3). In the present work, we have addressed these issues by analyzing signaling through the IR^{1152} mutant insulin receptor in cultured L6 skeletal muscle cells. The IR^{1152} receptor maximally activates metabolic responses in several cell types, preventing further stimulation by insulin (21, 34). In contrast, insulin mitogenic signals are normally mediated by this mutant (34), enabling us to investigate which receptor substrates are involved in proliferative and metabolic insulin effects. While the L6 muscle cells may not necessarily reflect all of the properties of skeletal muscle tissue *in vivo*, they have been widely used for studies on insulin action since they possess several characteristics of this tissue (21, 23–27). In addition, cultured cells with preserved IR substrate function provide an important tool for investigating the specific function of each substrate, complementary to the *in vivo*/*ex vivo* models. In fact, very recent data in knock-out animals have shown that disruption of IRS-1 gene results in compensatory mechanisms affecting the function of other substrates (20). These effects do not occur or may be more easily controlled in cultured cells with unaffected substrate expression. Furthermore, in the present study, L6 cell clones have been chosen expressing only small numbers of exogenous receptors. At these low levels of expression, abnormal cellular events that do not occur in the untransfected cells are unlikely to complicate the interpretation of our findings.

We report that IRS-2 was constitutively tyrosine-phosphorylated in L6 cells expressing the IR^{1152} receptor and did not undergo further phosphorylation following insulin exposure. At variance, in cells expressing the mutant receptor, IRS-1 phosphorylation exhibited little increase under basal conditions, but featured comparable insulin phosphorylation in cells expressing the wild-type and the IR^{1152} receptors. The differential phosphorylation of the two IRSs likely reflects an enhanced capability of IR^{1152} to bind the KRLB domain of IRS-2, independent of insulin. In fact, we have shown that (i) *in vitro*, IR^{1152} shows increased binding to the KRLB domain, which is only present in IRS-2; (ii) IR^{1152} normally binds to IRS-2 PTB domain and exhibits normal phosphorylation of the juxtamembrane NPEY motif (42), which is crucial for binding IRS-2 as well as IRS-1 and Shc PTB domains (39); and (iii) the differential phosphorylation of IRS-1 and IRS-2 by IR^{1152} occurs similarly *in vitro* and in intact cells, suggesting that it is not caused by discrete effects of the mutant receptor on the cellular mechanisms controlling phosphorylation of these substrates. Previous work has shown that binding of the KRLB domain requires phosphorylation of the tyrosine triplet in the insulin receptor regulatory domain (38), while phosphorylation of these residues is depressed in the IR^{1152} mutant (42). In the IR^{1152} receptor, however, we showed that the mutation mimics the effect of phosphorylation of the regulatory tyrosines (21), activating transduction of several biological effects in the absence of insulin. Thus, the constitutive IRS-2 binding to the regulatory loop of IR^{1152} may contribute to its unique signaling.

At variance with IRS phosphorylation, IR^{1152} was unable to phosphorylate Shc in intact cells, either in the absence or the presence of insulin. The absence of Shc phosphorylation in the Mut cells upon insulin exposure is consistent with the previously reported dominant activity of IR^{1152} over the small complement of endogenous IRs (34). However, the mechanism responsible for this defect does not seem to involve the inability of IR^{1152} to interact with Shc. In fact IR^{1152} binds Shc in a yeast two-hybrid system and phosphorylates it *in vitro*. Alternatively, we postulated that the abnormal cellular routing of the

mutant receptor (40) may impair its ability to bind Shc. Consistent with this hypothesis, in the present paper, we show that TPA treatment alters the cellular routing of wild-type insulin receptors, mimicking that of IR^{1152}, and, simultaneously, impairs Shc, although not IRS, phosphorylation. Previous studies also showed that IRS phosphorylation is unaffected by the inhibition of insulin and IGF-I receptor endocytosis (43, 44) while phosphorylation of Shc by the insulin and IGF-I receptors does appear to require receptor endocytosis (43, 44). Thus, the possibility exists that Shc phosphorylation occurs at an intracellular site distant from the plasma membrane and away from the IR^{1152} route.

In IR^{1152}-expressing clones, basal [^{3}H]thymidine incorporation is only slightly increased compared with that of cells expressing wild-type receptors, despite maximal IRS-2 phosphorylation and Grb2 association with IRS-2. On the other hand, insulin exposure simultaneously increased IRS-1 phosphorylation and Grb2 association, MAP kinase activity and [^{3}H]thymidine incorporation almost identically in mutant and wild-type cells. At variance with other cell types (8, 45), inhibition of PI 3-kinase activity with wortmannin did not impair at all insulin-induced DNA synthesis in L6 cells. It appears therefore that induction of the IRS-2·Grb2 complex is less efficient than that of IRS-1·Grb2 complex in transducing mitogenic responses and thus IRS-2 cannot substitute for IRS-1 in mediating the mitogenic action of insulin in Mut cells. In addition, these data indicate that the Grb2·SOS-activated MAP kinase cascade is a major pathway conveying insulin proliferative signals in these muscle cells via IRS-1 phosphorylation. Hence, preliminary experiments in our laboratory show that ribozyme suppression of IRS-1 in L6 cells and pre-exposure of the cells to the MAP kinase inhibitor PD98059 blocked insulin effect on thymidine incorporation. Consistent with our findings, Sharma *et al.* (46) have shown that adenovirus-mediated overexpression of IRS-1 interacting domains abolishes insulin-stimulated mitogenesis in 3T3-L1 adipocytes. Also, IRS-1-deficient mice exhibit growth retardation despite supranormal levels of IRS-2 phosphorylation in tissues (20) while IRS-2-deficient mice show progressive deterioration of glucose homeostasis with only small differences in growth (47). Finally, our recent work indicates that IRS-1 but not IRS-2 mediates IGF-I mitogenic responses (48), suggesting functional specialization in the IRS system. In most cells, including skeletal muscle cells, Shc also has an important function in transducing insulin effect on cell proliferation (15). However, our data show that the Shc-dependent component of the insulin mitogenic signal may be largely redundant in the L6 muscle cells, at least in those expressing the IR^{1152} mutant receptor. Hence, in these cells, full insulin activation of mitogenesis occurs in the absence of any detectable phosphorylation of Shc.

Different from thymidine incorporation, glycogen synthase activity and glycogen accumulation in $L6^{1152}$ cells were constitutively increased by the mutant receptor preventing further increase upon insulin exposure. A severely impaired increase in glucose disposal in response to insulin was also measured by us *in vivo*, in the skeletal muscle of diabetic individuals expressing the mutant receptor (49). It is possible therefore that insulin resistance in these patients is also contributed by a constitutive increase in muscle glycogen synthase activity and glycogen content due to the mutant receptor. The inhibition of PI 3-kinase activity blocked both the constitutively active glycogen synthesis in IR^{1152}-expressing cells and the insulin-dependent glycogen synthesis in cells expressing IR^{WT}. These data suggest that the same mechanism is involved in the control of the glycogen synthetic machinery by both the constitutively active IR^{1152} and the insulin-activated wild-type receptors. In the mutant cells, the glycogen synthetic process is fully activated concomitantly with the constitutive phosphorylation of IRS-2 but with almost no IRS-1 phosphorylation. In addition, it did not further increase upon insulin addition despite a 12-fold increase in IRS-1 phosphorylation. It appears therefore that IRS-2 mediates metabolic signaling in Mut cells. Consistent with this interpretation, very recent data (47) show that disruption of IRS-2 gene in mice leads to increased PI-3 kinase activity in IRS-1 precipitates from basal muscle tissue and nevertheless the IRS-2 knock-out mice are severely insulin-resistant. An alternative interpretation of our data is that dose responses for DNA synthesis and glycogen synthesis are quantitatively different in the L6 cells. Thus, only a small amount of phosphorylated IRS-1 (such as that present in the Mut cells) is sufficient to fully activate glycogen synthesis. In this event, however, one would expect that at submaximally effective receptor expression, IRS-1 should be rate-limiting for glycogen accumulation. In contrast, our data show that the expression of small numbers of IR^{1152} receptors are accompanied by undetectable levels of IRS-1 phosphorylation but still induce maximal IRS-2 phosphorylation and glycogen synthesis. Previous work in 3T3-L1 adipocytes showed that interference with the IRS-1-IR interaction did not cause inhibition of insulin-stimulated glucose transport, suggesting that alternate pathways exist in these cells (46). More recently, Zhou *et al.* (50) have shown that, in rat adipocytes, overexpression of high levels of IRS-1 as well as of IRS-2 increased basal and insulin-stimulated Glut4 levels in the plasma membranes indicating that both IRSs may signal Glut4 translocation when overexpressed in cells. Supramaximal IRS-2 phosphorylation or IRS-2 overexpression caused by IRS-1 deficiency may also be responsible for the residual insulin-stimulated glucose transport in soleus muscles of IRS-1 knock out mice (20). Here, we show that, even in the absence of absolute increases in phosphorylation or of overexpression, IRS-2 can mediate insulin metabolic effects in L6 cells.

In conclusion, we have provided evidence that IRS-2 mediates insulin regulation of glucose storage in the L6 cells expressing IR^{1152} receptors. In addition, IRS-1 and Shc activation of the MAP kinase cascade may be largely redundant in mediating proliferative responses in these cells.

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Differential Role of Insulin Receptor Substrate (IRS)-1 and IRS-2 in L6 Skeletal Muscle Cells Expressing the Arg $^{1152} \rightarrow$ Gln Insulin Receptor

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